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Continuous counter current extraction, isolation and determination of solanesol in *Nicotiana tobacum* L. by non-aqueous reversed phase high performance liquid chromatography☆

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Abstract

A method of continuous counter current extraction in a large-scale production of solanesol from tobacco leaves was developed. The crude extract containing 15–20% solanesol was subjected to a series of steps, viz., saponification, solvent recrystallization and column chromatography. The pure material was characterized by FT-IR, ESI-MS, ^{1}H NMR and ^{13}C NMR spectrometry. The analysis was carried out by a simple and rapid non-aqueous reversed-phase high performance liquid chromatographic method on a Hypersil BDS C_{18} column (250 mm × 4.6 mm, particle size 5 μm) with isopropyl alcohol–methanol (60:40, v/v) as mobile phase and detection at 215 nm. The product purity was between 95 and 98% (w/w) as determined by HPLC.

Keywords: Solanesol; *Nicotiana tobacum* L.; Counter current extraction; Isolation; Non-aqueous reversed-phase HPLC

1. Introduction

Solanesol a naturally occurring trisesquiterpenoid (C_{45}) alcohol of tobacco is one of the important precursors of the tumorigenic polynuclear aromatic hydrocarbons (PAHs) of tobacco smoke. Reduction of its levels in tobacco, leads to safe smoking products due to reduced PAH levels in cigarette smoke. It is also the starting material for many high-value biochemicals, including coenzyme Q10 and Vitamin-K analogues [1] as a starting material for Q10, it is used in treatment of different cancers. Coenzyme Q10 is well known not only to reduce the number and size of tumors but also improve cardiovascular health [2,3]. Solanesol itself could be used as an antibiotic, cardiac stimulant and lipid antioxidant. At present clinical trails are under progress to explore its use as an anticancer drug. There is a great demand for solanesol for production of Q10 and other uses. Thus, its isolation not only reduces the risks of PAH from tobacco smoke but also makes use of it as a starting material in synthesis of several value added products such as Q10 and other analogues. Therefore, isolation of solanesol from tobacco is gaining a lot of importance in recent years.

Solanesol is present in the lamina of tobacco leaves while absent from the stem and stalk [4]. The content of solanesol in tobacco depends upon a number of factors. It varies from 0.3 to 3.0% according to the type and variety of tobacco, duration of growth and method of curing [5,6]. A substantial portion of solanesol exists in the form of fatty acid esters due to which proper curing and saponification play an important role in converting them in to free solanesol [7]. Tobacco also contains several other organic compounds that can be easily co-extracted with solanesol and interfere with subsequent separation and purification processes [8]. One of the key problems in extraction of solanesol from tobacco is the selection of a suitable solvent for maximum yield and purity. As the solanesol lies in the cellular chloroplast of the tobacco leaves, not only the solubility but also penetrability of the solvent is very important for complete extraction. Further its separation from the

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crude extract and purification pose several problems because of the presence of closely related fatty acids, alcohols, alkaloids, tobacco pigments, tar and other organic impurities. The food and pharmaceutical grade solanesol has to be of highest purity for quality, safety and efficacy of the finished products. Therefore, it is quite important to develop processes that can selectively separate and purify solanesol from the crude extracts of tobacco leaves. Several methods were described in the literature for extraction, isolation and purification of solanesol from tobacco [9–13]. Most of the methods involve multiple step procedures, which are non-specific, quite tedious and time consuming. Generally, maceration, percolation, ultra sonication, soxhlet and bubble column were used for extraction of phytochemicals from the plant materials [14,15]. The first two techniques are not only time consuming but also give low yields of the desired products. Microwave-assisted extraction (MAE) coupled with saponification was reported to be effective for extraction of solanesol from tobacco leaves [16]. However, MAE and ultrasound sonication are useful for high value added products but consume high energy for commercial production. Soxhlet has limited analytical applications and not suitable for handling of bulk quantities of tobacco. Tang et al proposed an extraction procedure with petroleum ether under reflux at 50 °C followed by silica gel column chromatography for isolation and purification of solanesol from tobacco leaves [17]. However, the heat-reflux processes involve lengthy operations, bulk amount of solvents and ultimately thermal decomposition of the target compounds. Recently high-speed countercurrent chromatography (HSCCC) for isolation of solanesol from the crude extracts of tobacco was reported [18]. Here, the crude extract instead of raw tobacco was used as a feedstock for purification of solanesol. The purity of solanesol thus obtained was lees than 95%. A slow rotary counter current chromatography (SRCCC) involving a non-aqueous two-phase solvent system of sunflower oil-ethanol was also used to produce food grade solanesol in a commercial scale [19]. However, the process is not cost effective and the purity of solanesol was only 26%.

Reliable methods for determination of solanesol in tobacco are important for classification of different grades of tobacco according to their quality. A number of gas chromatographic methods for determination of solanesol in tobacco were reported [20–24]. The low volatility and poor FID response of solanesol render the technique unsuitable [20]. The sample preparation also involves a number of time-consuming derivatization steps [4,21,22]. Sheen et al. reported a packed column GC method involving lengthy extraction procedures and hydrogenation of solanesol [23]. The other methods include gravimetry [24] coulometry [25] and thin layer chromatography (TLC) [24]. HPLC with various detectors including UV, RID, ELSD and MS was used to determine solanesol. Most of the methods reported before 2006 were in normal phase mode with UV detection [26–28]. Until the early 2007, not even a single reversed-phase HPLC method for determination of solanesol in tobacco was reported. Recently, Zhoua et al. reported a RP-HPLC method using ELSD as a detector for determination of solanesol in tobacco [29]. ELSD is not only a specialized detector but also requires a large volume of nebulizer gas of high purity. It makes the method unsuitable for routine analysis solanesol because of the cost ineffectiveness. However, reversed phase HPLC with UV detection is often preferred not only because of its higher sensitivity but also wide availability and suitability. Quite recently, Chen et al. reported RP-HPLC with UV and ESI-TOF/MS determination of solanesol in the crude extracts of tobacco leaves [30]. The major drawback of this method lies in detection. The analytes were monitored at 202 nm by PDA where the acetonitrile used as one of the mobile phase solvents generally interferes. The use of such a short wavelength UV also produces baseline artifacts. Due to the increasing demand for solanesol, a convenient and rapid method for determination as well as isolation of solanesol from tobacco is highly needed.

In the present investigation: (i) a simple protocol involving the use of a continuous counter current extraction followed by saponification, solvent crystallization or column chromatography for isolation of solanesol from *Nicotiana tobacum* L. was developed. It was compared with soxhlet extraction in terms of efficiency and recovery. Further, a simple non-aqueous RP-HPLC with UV method for determination of solanesol in *Nicotiana tobacum* L. was described.

2. Experimental

2.1. Materials and reagents

All the reagents were of analytical-grade unless stated otherwise. HPLC-grade isopropyl alcohol and methanol (Ranbaxy, SASNagar, India) were used. Dried tobacco received from local industries in Hyderabad, India and farmers of Nigeria, West Africa were used.

2.2. Counter current extraction

The counter current continuous extraction was carried out in nine stages. Initially the dried leaves were powdered in pulveriser and particles of 2–3 mm size were pelletized of size 12 mm × 24 mm using steam. The pellets were then passed to the charge hopper of continuous extractor with the help of a bucket conveyor by means of gravity. Hexane was fed to the extractor from the storage tank. Later the solvent was recovered from the extract and recycled. The crude extract contains 15–20% solanesol. The raffinate was disposed after the recovery of solvent. Finally the solanesol-enriched hexane was collected in miscilla tank. The miscilla extract was send to multiple vacuum evaporators for solvent recovery with the help of a pump. The accumulated water in miscilla tank was separated and drained off as an effluent. In each stage the recirculation pumps were kept in running at a flow rate of 10 l/s to extract the solanesol from the pellets.

2.2.1. Extraction conditions

Flow rate of the feed (pellets): 2.5 t/h, residence time: 4 h, flow rate of the circulating solvent: 6 kl/h, extractor still volume: 16 kl, ratio of solvent to solid feed: 2:4, extraction temperature: 60 °C and pressure: 600 mmHg.

2.2.2. Process operation parameters

Number of stages: 9, pump capacity: 10 lps, extractor dimensions (HLW): 1.5 × 14 × 1.5, total volume of extractor: 31.5 m^3, bulk density: 0.5–0.65 kg/l, bed height: 0.75 m, vapour space: 50%, quantity in each stage except feed: 1.75 m^3, total quantity processed/hour: 2.56 t (with moisture), extract: 3.07 t/day (20% purity), extract: 0.62 t/day (100% solanesol), extraction temperature: 60 °C. The extract was dissolved in methanol, sonicated for 10 min, filtered and analyzed by HPLC.

2.3. Soxhlet extraction

Twenty-five grams of dried and powdered tobacco was placed in 250 ml soxhlet thimble and fitted with 500 ml round bottom flask containing 250 ml hexane and refluxed for 4 h. The contents were cooled and the hexane was removed on a rotary evaporator. It was dissolved in methanol, sonicated for 10 min, filtered and analyzed by HPLC.

2.4. Isolation/purification

About 5 g of the crude extract was saponified with 120 ml of 0.5 N methanolic potassium hydroxide. The mixture was refluxed for 2 h. After cooling, the mixture was filtered through Whatmann No. 42 filter paper into a separating funnel and the flask and filter were washed with 50 ml of methanol. Hexane (150 ml), 75 ml of aqueous saturated KCl solution and 150 ml of water were added vigorously shaken to effect solvent partitioning. The hexane layer was removed and the aqueous layer was extracted with hexane (2× 50 ml). The hexane extracts were combined, washed with water, reduced the volume on a rotary evaporator and analyzed by HPLC (∼45%). About 0.3 g of the sample was loaded on 30 g of 100–200 mesh silica gel and placed on a column (50 cm × 2 cm i.d.). The column was eluted with 5% (v/v) ethyl acetate in hexane. The eluent was collected in 20 ml fractions. Fractions containing solanesol were monitored with the help of HPLC and all the similar fractions concentrated under vacuum at 40 °C to obtain a pale yellow residue. The residue was crystallized by dissolving in hexane, stored at −20 °C and analyzed by RP-HPLC.

2.5. Apparatus

The HPLC system was composed of two LC-10AT VP pumps, one LC-8A pump, an SPD-10AVP diode array detector an SIL-10 AD VP auto injector, a DGU-12 A degasser and SCL-10A VP system controller (all from Shimadzu, Kyoto, Japan). A reverse-phase Hypersil column (250 mm × 4.6 mm i.d., particle size 5 μm) was used for separation and determination. The chromatographic and integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system. The ^1H and ^{13}C NMR spectra were recorded using Brucker 300 MHz (Varian, Palo Alto, USA) FT-NMR spectrometer containing $^1H/^{13}C$ dual probe. A 5 mm glass tube was used to place the sample in to the magnetic field of the spectrometer under the following conditions: ^1H NMR; resonance frequency 300 MHz, spectral width 6188 Hz, pulse width 5.8 μs, data points 16,834, spectral resolution 0.3 Hz, probe temperature 27 °C. ^{13}C NMR; resonance frequency 75.46 MHz, spectral width 17,985 Hz, pulse width 6.25 μs, data points17,982, spectral resolution 3 Hz, probe temperature 27 °C. ^1H and ^{13}C chemical shifts were reported in ppm, relative to tetra methyl silane (δ 0.0; TMS). $CDCl_3$ (δ 77.0) was used as an internal standard. The IR spectra were recorded using a Fourier-transform infrared spectrometer (Thermo Nicolet, Nexus 670, USA) range 4000–400 cm^{-1} and resolution 4 cm^{-1}. The numbers of scans were 20. The spectra were recorded in the solid state using a 1% KBr pellet. The instrument was calibrated by using a polystyrene standard. ESI-MS spectra were recorded using a Micromass Quatro mass spectrometer (Micromass, UK) equipped with electro spray interface. Nitrogen was used as a nebulizer and dissolution gas. Nebulizer gas flow 0.9 l/min, dissolution gas flow 9.8 l/min, nebulizer pressure 50 psi, capillary voltage 3.0 kV, cone voltage 25 V, source block temperature 20 °C, ion energy 2.0 V. Mass measurements were performed in the full-scan mode over the mass range of *m/z* 50–1000 with 0.21 scans/s.

2.6. Chromatographic conditions

Analytical HPLC was performed with Hypersil BDS C_{18} (Thermo Electron corporation) column (250 mm × 4.6 mm i.d., particle size 5 μm) using two LC-10AT VP pumps. Before delivering in to the system, the mobile-phase consisting of IPA: MeOH (60:40, v/v) was filtered through 0.45 μm, PTFE under vacuum and degassed by purging with helium. The analysis was carried out under isocratic conditions using a flow rate of 0.7 ml/min at room temperature (28 °C). Chromatograms were recorded at 215 nm using SPD-10A VP photodiode array detector.

3. Results and discussion

3.1. Extraction of solanesol

It is always economical to produce solanesol first in the form of a crude extract in a continuous counter current extractor. The extract was further purified to high-grade solanesol by different methods. In the counter current extraction process, steam was introduced in to the pellitiser so that, the powder absorbs around 5–8% moisture while forming the pellets. The moisture helps in the separation of water-soluble compounds and increases the content of solanesol in the extract. The water was finally collected in the miscilla tank and disposed off in the form of an effluent. The bulk density of the powder and the pellets were almost same as 0.5–0.6 kg/l. At every stage of the counter current continuous extractor, the feed solvent, i.e. hexane was enriched with solanesol and its concentration increased till it reached the miscilla tank. In other words concentration of solanesol in pellets came down as it moved from stages 1 to 9. There by a concentration gradient was maintained to help in the mass transfer between the pellets and the solvent. During the preliminary trails, the loss of solvent was 0.5–1.0%. It was observed that 4 h of extraction gave a maximum yield of 5–6% of solvent. Thus, the residence time was fixed at 4 h. Bed heights were fixed on the basis of

amount of leaf powder/pellets to be processed. The hexane was removed from the extract by multiple evaporators and analyzed by RP-HPLC.

3.2. Effect of particle size

The effect of particle size on the extraction yield of solanesol was studied with leaf particle size varies from 2.83 to 9.51 mm. It was observed that the yield of solanesol increased when the particle size of the tobacco was small. The small size of the particles increases the surface area of tobacco dust. Therefore, it is easier to extract solanesol from tobacco having small particle size. Thereby, the optimized particle size of leaf for extraction of solanesol by continuous counter current extraction was <3 mm.

3.3. Effect of solvent

The effect of different solvents, viz., hexane, trichloromethane, acetone, methanol and ethanol on the yield of solanesol was investigated. All the solvents have good solubilization ability of solanesol. Even though methanol had a good extraction capacity, keeping in view of the economics, hexane was found to be the best alternative for industrial scale since its latent heat was three times less than that of methanol. This has not only minimized the consumption of energy but also enhanced the applicability of the process for commercial production.

3.4. Effect of time

The optimum time for extraction was determined by analyzing the sample in different time intervals 0–6 h. It was observed that the extraction for 4 h gave a maximum yield of 5–6% solanesol. The dependence of recovery of solanesol on time of extraction is shown in Fig. 1. The counter current extraction was completed within 4 h after which there was no further improvement in the yields.

3.5. Purification of solanesol

The crude extract containing 15–20% of solanesol was purified by three different methods: (i) silica gel column chromatography; (ii) saponification followed by silica gel column chromatography; (iii) saponification followed by recrystallization with different solvents, viz., acetone, methanol and acetonitrile. In the first method, about 0.3 g of the sample was loaded on 30 g of 100–200 mesh silica gel packed in a column of 50 cm × 2 cm i.d. The column was eluted with 5% (v/v) ethyl acetate in hexane. All the similar fractions were concentrated under vacuum at 40 °C to obtain a pale yellow residue. The solanesol thus obtained was 90–93% pure. It was further recrystallized with hexane to improve the purity to 95%. In the second method, the crude was initially saponified by methanolic KOH to get 45% of solanesol which was further purified by silica gel column to 95%. The product was further purified by hexane crystallization. The purity was 98% with a good yield of 2.1%. In the third method, the saponified crude of 45% solanesol was dissolved in warm acetone (10 ml/g) and kept in refrigerator for 72 h and the crystals formed were removed by filtration and analyzed, where the solanesol was found to be around 70%. The purity of solanesol was further improved to >90% when it was recrystallized in methanol and twice in acetonitrile. Of the three methods, silica gel column purification of crude solanesol and hexane recrystallization gave about 1.8% yield of the product. In the second method saponification followed by column purification gave good yield of 2.1% and more than 98% purity with hexane recrystallization. In the third attempt the saponified fraction was subjected to recrystallization with common laboratory solvents gave the yield 0.8%. This could be due to the fact that a substantial portion of the solanesol in tobacco exists in the form of esters of fatty acids rather than free solanesol. Saponification of the crude converts the solanesyl esters to solanesol and a series of fatty acids. Therefore, the saponification of the crude extract increased the solanesol content and in turn the yield. Hence, it is a best choice for isolation and purification of solanesol from tobacco. The analysis results of the counter current, soxhlet extracts and the purified fractions of solanesol are given in Table 1.

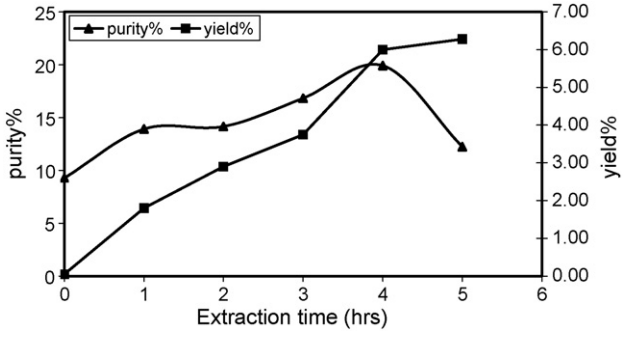

Fig. 1. Effect of extraction time on the yield and purity of solanesol in counter current extraction.

3.6. Spectral data

The isolated solanesol was identified by the following spectral data. IR: 3378, 2965, 2917, 2851, 1664, 1446, 1382, 1151, 1104, 992, 875, 837, 795, 750, 599. MS (ESI) *m/z* 631 (*M* + H), ^{1}H NMR (200M$_Z$) (solvent: $CDCl_3$) δ ppm: 1.36 (3H, 1CH_3), 1.59 (21H, 7CH_3), 1.66 (s, 3H) 1.68 (s, 3H), 1.90–2.12 (m,

Table 1
Results of analysis of crude and purified extracts of solanesol by RP-HPLC

Sample	Solanesol content (%, w/w)	
	Counter current	Soxhlet
Crude extract	21.01 ± 0.03	17.32 ± 0.05
Saponified	45.01 ± 0.02	43.43 ± 0.07
Acetone RC	70.02 ± 0.03	66.09 ± 0.03
Acetonitrile RC	91.45 ± 0.02	90.34 ± 0.04
Silica gel column	93.51 ± 0.01	92.53 ± 0.03
Saponified + silica gel column	96.32 ± 0.04	95.58 ± 0.05
Silica gel column + hexane RC	98.05 ± 0.05	96.12 ± 0.05

RC: recrystallization.

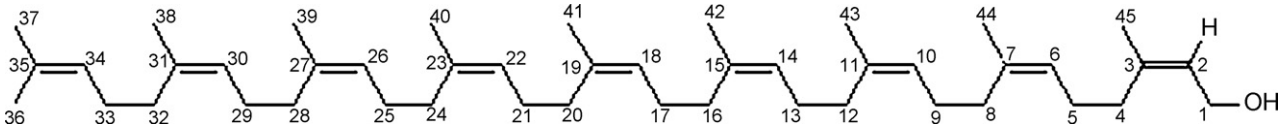

Fig. 2. Chemical structure of solanesol.

32H), 4.10 (m, 2H,O–CH_2), 5.01–5.12 (t, 8H) 5.39 (t, 1H). ^{13}C NMR (200M_Z) (solvent: $CDCl_3$) δ ppm: 15.97 (C38–C44), 16.19 (C37), 17.60 (C45), 25.60 (C36), 26.70 (C8, C12, C16, C20, C24, C28, C32), 26.80 (C33), 29.67 (C4), 39.70 (C9, C13, C17, C21, C25, C29), 59.29 (C1), 123.51–124.46 (C2, C34, C30, C18, C22, C26, C6, C10, C14), 131.03 (C35) 134.80–135.31 (C31, C19, C23, C27, C7, C11, C15), 139.49 (C3). The chemical structure of solanesol is shown in Fig. 2

3.7. HPLC method development

Solanesol, the C_{45} terpenoid of the lipid soluble fraction of tobacco is soluble in polar organic solvents but insoluble in water. Thus, silica was the preferred stationary phase for analytical separations. However, in normal phase mode, solvents of low polarity must be used to achieve adequate retention. But it becomes quite difficult to maintain reproducibility in such systems unless the trace amounts of water in the solvents are carefully controlled. Such problems encountered in normal phase chromatographic separation of hydrophobic compounds are generally overcome by reversed-phase chromatography. The retention of hydrophobic compounds on chemically bonded C_{18} phases is generally large and non-aqueous solvents such as methanol, acetonitrile and THF should be used to accomplish the elution in an acceptable time. For example the separation of fats, carotinoids and sterols have been generally carried out by non-aqueous reversed phase chromatography [31,32]. Under such conditions the homologues/isomers are better resolved than on silica, which is another advantage of reversed-phase separations. Thus, non-aqueous reversed phase HPLC was carried out to separate solanesol effectively from other components of tobacco. Hypersil BDS C_{18} column (250 mm × 4.6 mm i.d., particle size 5 μm) with a mixture of isopropyl alcohol–methanol using a UV at 215 nm was used. The mobile phase composition was optimized and it was found to be IPA:MeOH (60:40, v/v) for better separation. The total run time between the injections was 15 min. Identification of solanesol was based on co-injection and comparison of retention time with that of a standard. The HPLC chromatograms of solanesol (a) extracted by counter current extraction (b) purified by different methods are shown in Figs. 3 and 4, respectively.

3.8. Validation

3.8.1. Linearity

Calibration was carried out in the range of 0.1–1.25 mg/ml. The mean equation of the calibration curve (n = 6) obtained from six points was $y = 13{,}473{,}671x + 1{,}560{,}765$ with a regression coefficient of 0.9996.

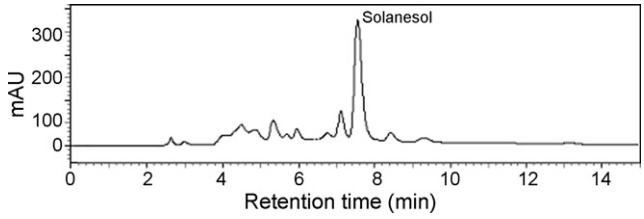

Fig. 3. HPLC chromatogram of a continuous counter current extract of solanesol from *Nicotiana tobacum* L.

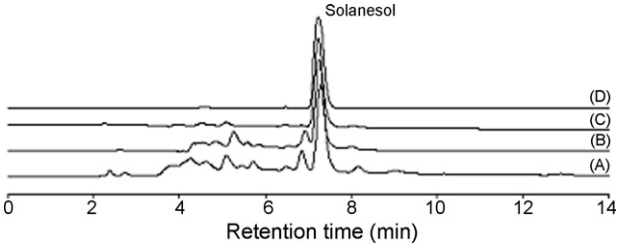

Fig. 4. HPLC profiles of (A) crude extract; (B) saponified; (C) saponified and acetone RC; (D) silica gel column and hexane RC.

3.8.2. Accuracy and precision

The precision was evaluated by repeated injections of the sample solution six times. The R.S.D. of peak area and retention time was 1.6%. Intra- and inter-day variabilities were determined by analysis of standard solutions at low, medium and high concentrations of solanesol on three different days. The acceptable intra- and inter-day precisions (R.S.D.) and accuracy (relative error, RE) were <1% and ±5%, respectively. The assay precision was <5%, and the accuracy was >98%.

3.8.3. Limits of detection (LOD) and quantification (LOQ)

LOD was defined as the lowest concentration of solanesol at which the signal was larger than three times of the baseline noise S/N = 3 and LOQ as S/N = 10. The measured LOD and LOQ values were 0.2 and 0.7 μg/ml, respectively.

Table 2
Content of solanesol in different raw materials of tobacco of Nigeria as determined by RP-HPLC

Tobacco	Solanesol content (%, w/w)
Fibers	0.63 ± 0.01
Mody	0.46 ± 0.01
Dust	0.44 ± 0.02
Sweeding	0.47 ± 0.02
Wet offals	0.65 ± 0.02
Top leaf	1.00 ± 0.01
Middle leaf	1.01 ± 0.02
Lower leaf	0.43 ± 0.03

3.9. Applications

The developed RP-HPLC method was used for determination of solanesol in tobacco of different grades obtained from local and Nigerian farmers. The content of solanesol in different raw material of tobacco was determined and the results are given in Table 2. The method showed efficient separation of solanesol from different components of tobacco.

4. Conclusions

An economical and efficient protocol for isolation of solanesol from tobacco using counter current extraction, followed by column chromatography, saponification and recrystallization was described. High purity of 95–98% solanesol was produced using common laboratory chemicals. The continuous counter current extraction is more suitable for isolation of solanesol on a large scale. In addition, a simple and rapid method for separation and determination of solanesol from tobacco using non-aqueous RP-HPLC in an isocratic elution mode and using UV detector at 215 nm was developed. The non-aqueous reverse-phase mode has definite advantages over other methods due to the use of most popular C_{18} column with UV detection is often preferred not only because of its higher sensitivity but also wide availability and suitability.

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